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Secreted and Placental Membrane Forms of Folate-Binding Protein Occur Sequentially During Pregnancy in Swine[1]

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ABSTRACT

The objective was to understand how two forms of folate-binding protein interact to accomplish folate transport during pregnancy in swine. Specific folate binding was measured in uterine flushings during the estrous cycle and early pregnancy and in allantoic fluid (secreted form) and placental membranes (membrane form) throughout later pregnancy. In addition, the localization of the secreted form of folate-binding protein (sFBP) in uterine wall sections was assessed. Uterine flushings were collected on Days 10, 13, and 15 of the estrous cycle and pregnancy. Allantoic fluid and placentas were collected on Days 20, 35, 50, 70, 90, and 105 of pregnancy. Uterine-wall sections were collected on all days of the experiment. Folate binding was measured by incubation of aliquots of uterine flushings, allantoic fluid, or placental microsomal membranes with 0.5–4 nM [^{3}H]folate. Uterine-wall sections were incubated with purified anti-FBP IgG or normal rabbit serum IgG to localize sFBP. Folate binding did not differ between early pregnancy and the estrous cycle in uterine flushings, was greatest from Day 50 to 70 of pregnancy in allantoic fluid, and was greatest from Day 50 of pregnancy onward in placental microsomal membranes. Staining for sFBP was present in the endometrial glands from Day 10 to 15 in cyclic gilts and from Day 10 to 20 in pregnant gilts. The pattern of folate binding and sFBP staining supports the concept that sFBP transports folate to the developing conceptus until placentation and then the placental form takes over folate transport.

conceptus, early development, embryo, endometrium, placenta, uterus

INTRODUCTION

Folic acid is a vitamin that is required as a cofactor in the transfer of methyl groups [1]. Metabolites of folic acid are required for synthesis of the purine ring, methionine and thymidine, which are all essential for cell division in and growth of [2] the developing fetus. Deficiencies in folate lead to abnormal erythropoiesis [3] and birth defects [4]. Previous results suggest that the amount of folate available to the developing conceptus influences fetal erythropoiesis [5] and fetal erythropoiesis influences uterine capacity of swine [5–7]. In addition, it has been reported that folate supplements increased the survival rate of fetuses during early gestation [8]. However, supplements of folic acid resulted in equivocal effects on litter size in swine, increasing litter size in some studies [9–11], but having no effect in another [12]. Taken together, these studies suggest that folate transport to the developing swine conceptus may influence the reproductive success of swine.

It is not fully understood how folate transport is controlled during pregnancy. Folate-binding activity is present in the uterine flushings [13] and the uterus secretes a folate-binding protein (FBP) that is likely to be involved in folate transport to the developing conceptus [14, 15]. However, the secretion of FBP beyond Day 15 of pregnancy has not been investigated. In addition to secreted FBP (sFBP), a cDNA and gene corresponding to a placental membrane form of FBP (mFBP) have been cloned and sequenced [16, 17]. Both forms of FBP are expressed in the intrauterine environment during pregnancy in swine [16] and thus may be important for folate transport to the conceptus during pregnancy. The objectives of this study were to 1) compare specific folate binding during the estrous cycle and early pregnancy in uterine flushings and in allantoic fluid and by placental microsomal membranes during later pregnancy and 2) determine the localization of sFBP in the intrauterine environment throughout pregnancy.

MATERIALS AND METHODS

Experimental Animals

All procedures described in this manuscript were reviewed and approved by the MARC Institutional Animal Care and Use Committee. White composite (¼ Landrace, ¼ Yorkshire, ¼ Large White, and ¼ Chester White) gilts were observed for at least one estrous cycle of normal length (17–23 days) and were then inseminated. Gilts were killed on Days 10, 13, or 15 of the estrous cycle or pregnancy (n = 4–6 per day). The uterus was flushed with minimum essential medium containing a reduced amount of folate (50 ng/ml). After centrifugation to remove cells, debris, and conceptus, uterine flushings were frozen and stored at −60°C until they were used for folate-binding analysis. Additional White composite gilts (n = 63) were slaughtered on Days 20, 35, 50, 70, 90, and 105 of pregnancy (n = 3–6 per day). For these gilts, allantoic fluid (10 ml) was collected from three conceptuses per gilt except on Day 20 (due to a low volume, allantoic fluid samples were pooled from conceptuses on Day 20). After centrifugation to remove cells and debris, allantoic fluid samples were frozen at −60°C until used for folate-binding analysis. Placenta (2 g) samples were collected from two conceptuses at random for each gilt on Days 35, 50, 70, 90, and 105 of pregnancy for microsomal membrane preparation (there was not enough placental tissue on Day 20 for microsomal membrane preparation). A section of the uterine wall was collected on all days of the experiment and fixed with 4% paraformaldehyde in PBS. After 16 h, fixed tissues were washed with PBS (2 × 1 h), dehydrated through a graded series of ethanol concentrations (1 × 20% 1 h, 1 × 40% 1 h, 1 × 60% 1 h, 1 × 70% 1 h, 1 × 95% 1 h, 2 × 100% 1 h), followed by three changes of xylene (1 h each). After two changes of paraffin at 60°C (1 h each), tissues were embedded in paraffin.

Sample Preparations for Folate-Binding Analysis

Uterine flushings and allantoic fluid samples were dialyzed against 50 mM glycine, pH 2.8, to remove endogenous folate bound to sFBP, fol-

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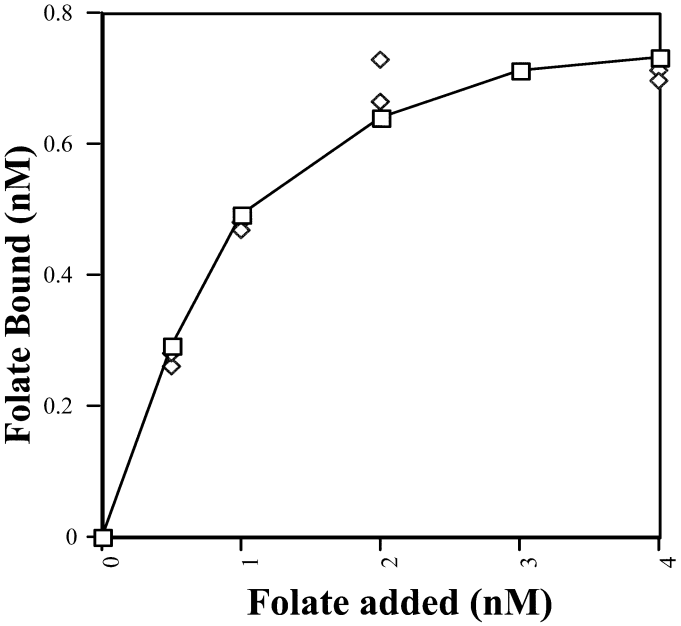

FIG. 1. A representative saturation curve of allantoic fluid folate-binding protein. The line and squares represent the predicted saturation curve based on B_{max} and K_d values calculated from nonlinear regression of the observed data using the formula (specific bound = [B_{max} × amount added-specific bound]/[K_d + amount added-specific bound]). Diamonds represent the actual values measured.

lowed by dialysis against PBS, pH 7.4. Uterine flushings (10 μl of 1:10 dilution) and allantoic fluid samples (10 μl) were then incubated with varying concentrations of [^{3}H]folate (Amersham Biosciences, Piscataway, NJ) ranging from 0.5 to 4 nM for 16 h at 4°C in the absence or presence of excess unlabeled folate (1 μM) in a total volume of 0.4 ml in PBS containing 0.25% bovine serum albumin. A preliminary experiment indicated that folate binding was stable after 1–24 h incubation, indicating equilibrium was achieved. There were no differences in binding when uterine flushings were incubated with [^{3}H]folate for 1, 2, 4, 8, and 24 h. Free folate was removed by incubation with 0.4 ml 0.25% charcoal, 0.025% dextran in 50 mM Tris, pH 8.2, for 15 min followed by centrifugation at 1000 × *g* for 10 min. An aliquot (0.5 ml) of the resulting supernatant was then used for scintillation counting (Fig. 1). For placental microsomal membranes, a crude membrane preparation was prepared according to Spencer et al. [18]. Placental tissue samples (1 g) were homogenized by polytron in 5 ml of PBS containing 100 mM PMSF, 2 μg/ml antipain, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A. After centrifugation at 1000 × *g* for 10 min, the supernatant was collected. Then samples were centrifuged at 100 000 × *g* for 1 h and the pellet was collected. Pellets were resuspended in 50 mM glycine, pH 2.8, to remove endogenous folate bound to mFBP and kept on ice for 10 min in a 1.5-ml tube. Then samples were centrifuged for 10 min on a microcentrifuge at 13 000 rpm and the supernatant was removed. The pellet was resuspended in PBS (pH 7.4) and sonicated to disperse placental microsomal membranes. Protein concentration was measured [19] and 25 μg of placental microsomal membrane protein in 100 μl of PBS was then incubated with varying concentrations of [^{3}H]folate as described above for uterine flushings and allantoic fluid samples. Bound folate was separated from free folate by centrifugation at 1000 × *g* for 30 min, 300 μl was removed, and the remaining 100 μl and the pellet were washed with 1 ml of PBS. The samples were centrifuged again (1000 × *g*) for 30 min and 1 ml was removed. Pellets were mixed with 2 ml of scintillation cocktail, vortexed, and the amount of radioactivity in the samples was determined by scintillation counting.

Immunohistochemistry

Uterine wall sections (6 μm) were deparaffinized in xylene (2 × 5 min; Sigma Chemical Co., St. Louis, MO) and rehydrated to water through a graded series of ethanol concentrations (2 × 100% 2 min, 2 × 95% 2 min, 1 × 70% 5 min). Antigenic sites were revealed by heating the sections in 50 mM Tris, 0.1% SDS, and 1% β-mercaptoethanol. Endogenous peroxidase activity was quenched by incubating the sections for 30 min in 0.3% hydrogen peroxide. Sections were then incubated with buffer (50 mM Tris, 0.5 M NaCl, and 1% Triton X-100) for 30 min to block nonspecific binding. Then sections were incubated with 100 μg/ml of either purified rabbit anti-porcine FBP [14] IgG or normal rabbit serum IgG. Both IgGs were purified using a protein A kit (Sigma Chemical Co.). Localization of bound IgG was determined using the Vectastain Elite ABC reagent with DAB as chromogen (Vector Laboratories, Burlingame, CA). Tissue sections were counterstained with hematoxylin and then dehydrated through a graded series of ethanol concentrations and xylene. Tissue sections were mounted with DPX mounting media (Fluka Biochemica, Steinheim, Germany).

TABLE 1. Folate binding (B_{max}) in the uterine flushings.

Day	Least squares means (nmoles/μl uterine flushings)	Standard error means (range)[a]
10	0.058	0.0352, 0.0946
13	3.07[b]	2.094, 4.504
15	6.34	4.384, 9.171

[a] Data were log transformed for analysis. Least squares means and standard error means were calculated by taking the antilog of the log-transformed least squares means and standard error means. Range is the least squares mean ± SEM.
[b] Day 10 different ($P < 0.01$) from Days 13 and 15, which were not different from each other.

Statistical Analysis

The B_{max} and K_d values for uterine flushings, allantoic fluid, and placental microsomal membranes for each gilt were generated using nonlinear regression analysis (model: specific bound = [B_{max} × free]/[K_d + free]). These values were then subjected to analysis of variance using the general linear models procedures of the statistical analysis system. The model used to analyze folate binding in uterine flushings for the estrous cycle and early pregnancy included day of the estrous cycle or pregnancy, status, and day × status interaction. The effect of day on uterine flushing samples was further examined using the following contrasts: 1) Day 13 vs. Day 15 and 2) Day 10 vs. Day 13 and Day 15 combined. The model used to analyze folate binding in allantoic fluid and placental microsomal membrane samples for later pregnancy included the effect of day of pregnancy. These data were further examined using the following contrasts: 1) Day 35 vs. Day 50, 2) Day 50 vs. Day 70, 3) Day 70 vs. Day 90, and 4) Day 90 vs. Day 105.

RESULTS

In uterine flushings, there was a significant ($P < 0.01$) day effect on the concentration of folate-binding sites (B_{max}), but no significant status effect or day × status interaction was observed. Folate-binding sites (B_{max}) increased ($P < 0.01$) from Day 10 to Day 13 and did not change between Days 13 and 15 (Table 1). The affinity for folate binding in uterine flushings (K_d) did not differ between the days measured (overall least squares means ± SEM of K_d's for cyclic and pregnant gilts were 0.25 ± 0.07 nM and 0.33 ± 0.09, respectively). On Day 20, B_{max} and K_d were too low to measure in allantoic fluid. In addition, B_{max} increased ($P = 0.01$) from Day 35 (0.063 ± 0.023 nmoles bound/μl allantoic fluid) to Day 50 (0.158 ± 0.026), did not differ between Days 50 and 70 (0.141 ± 0.023), decreased ($P < 0.01$) on Day 90 (0.026 ± 0.02), and did not differ between Days 90 and 105 (0.01 ± 0.019; Fig. 2A). The K_d did not differ between the days measured (overall least squares mean ± SEM of K_d was 0.19 ± 0.081 nM) (Fig. 2B). In placental microsomal membranes, B_{max} increased ($P < 0.01$) from Day 35 (9.2 ± 2.4 nmoles bound/mg microsomal membrane proteins) to 50 (21.2 ± 2.8). B_{max} did not differ between Days 50 and 70 (14.4 ± 2.4), Days 70 and 90 (10.8 ± 2), and Days 90 and 105 (14.8 ± 2) (Fig. 3A). The K_d in placental microsomal mem-

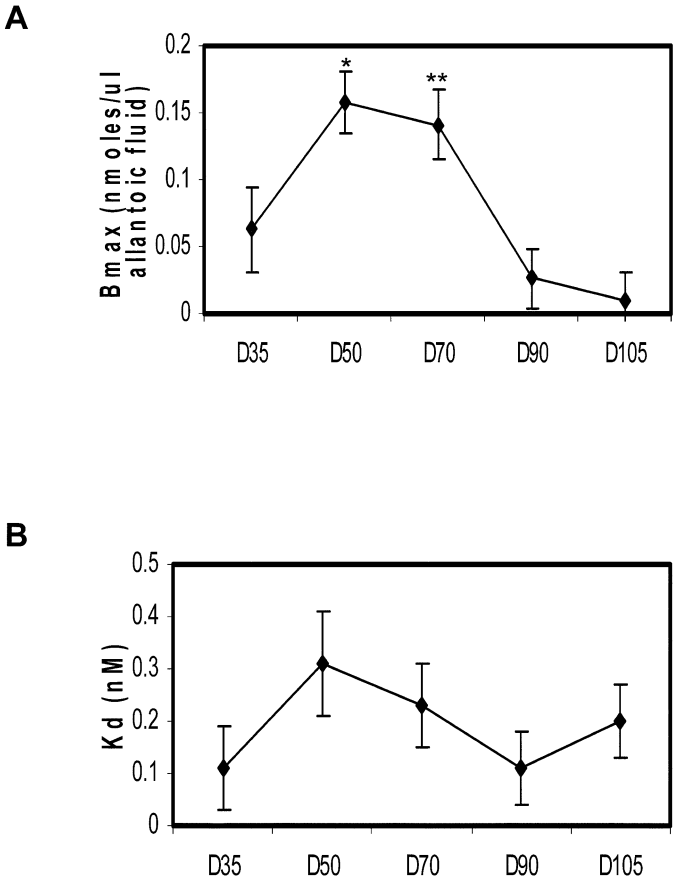

FIG. 2. **A**) Least squares means (± SEM) for the concentration of folate-binding sites (B_{max}) in allantoic fluid from pregnant gilts. The maximum folate binding occurs between Days 50 and 70 and then declines. Day 50 is different (*$P = 0.01$) from Day 35 and Day 70 is different (**$P < 0.01$) from Day 90. **B**) Least squares means (± SEM) for the K_d for folate binding in allantoic fluid. The K_d did not differ between the days measured.

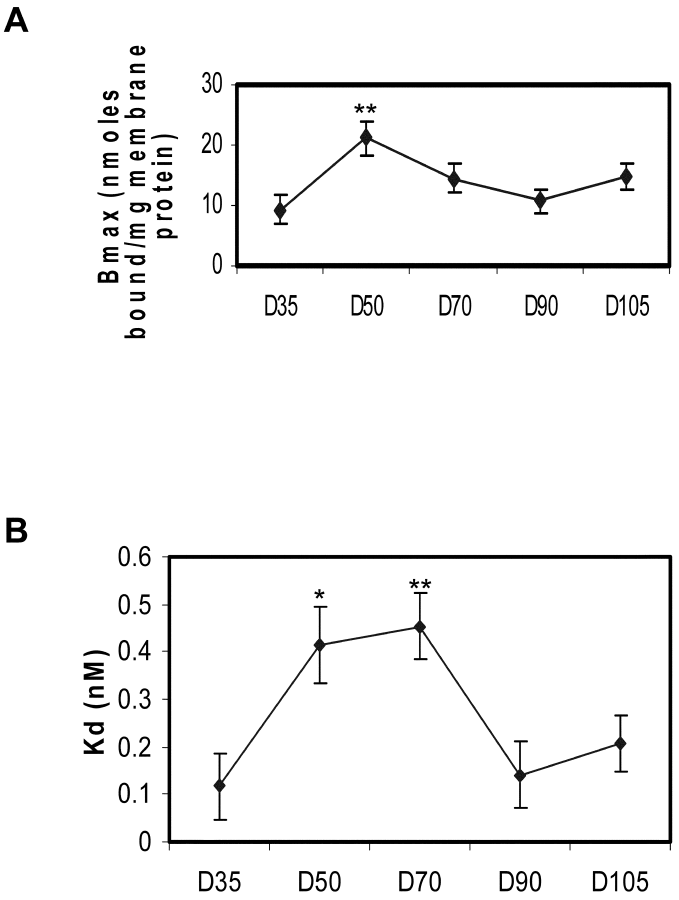

FIG. 3. **A**) Least squares means (± SEM) for the concentration of folate-binding sites (B_{max}) in placental microsomal membranes from pregnant gilts. Folate-binding sites increased (**$P < 0.01$) on Day 50 and after that did not change significantly through Day 105. **B**) Least squares means (± SEM) for the K_d for folate binding in placental membranes. The K_d was greater (i.e., decreased affinity) between Days 50 and 70 compared with the other days measured. Day 50 is different (*$P = 0.02$) from Day 35 and Day 70 is different (**$P < 0.01$) from Day 90.

branes increased ($P = 0.017$) from Day 35 (0.12 ± 0.07 nM) to Day 50 (0.41 ± 0.08), did not differ between Days 50 and 70 (0.45 ± 0.07), decreased ($P < 0.01$) between Days 70 and 90 (0.14 ± 0.07), and did not differ between Days 90 and 105 (0.21 ± 0.06) (Fig. 3B).

In cyclic gilts, light staining for sFBP was present in the endometrial glands on Day 10, appeared to be stronger on Day 13, and appeared to decrease by Day 15 (Fig. 4, A and B). In pregnant gilts, light staining for sFBP was present in the endometrial glands on Day 10 and appeared to be stronger on Days 13, 15, and 20. The sFBP staining was absent after Day 20 of pregnancy (Fig. 4, A and B). There was no staining for sFBP in the placenta throughout pregnancy.

DISCUSSION

This is the first experiment to compare the folate-binding characteristics of uterine flushings, allantoic fluid, and placental microsomal membranes during pregnancy in gilts. In uterine flushings, folate binding increased between Days 10 and 13, with no differences in either amount of binding or affinity of binding between the estrous cycle and early pregnancy. In allantoic fluid, maximum folate binding occurred between Days 50 and 70 of pregnancy and then declined, while the affinity did not change. In placental microsomal membranes, maximum folate binding occurred on Day 50 and did not change between Days 50 and 105, while the affinity was lowest between Days 50 and 70. In addition, staining for sFBP was localized in the endometrial glands from Day 10 to Day 15 of the estrous cycle and from Day 10 to Day 20 of pregnancy.

The increase of B_{max} in uterine flushings between Days 10 and 13 of pregnancy coincides with the elongation of the porcine conceptus [20], and sFBP in the uterus likely transports folate to the rapidly developing conceptus. The elongating porcine conceptuses secrete estrogen into the lumen of the uterus on Days 11 and 12 of pregnancy [20]. However, cyclic gilts, without estrogen secretion into the lumen of uterus from elongating conceptuses, have similar folate-binding activity compared with pregnant gilts, as shown in this study and previous studies [14, 15]. This indicates that production of sFBP is not controlled by conceptus estrogen secretion. Rather, progesterone given on Days 2 and 3 after estrus accelerated the increase in intrauterine sFBP content, suggesting that duration of progesterone may be the primary factor controlling the onset of sFBP production [15].

In allantoic fluid, maximum folate binding occurs between Days 50 and 70 of pregnancy and then declines, while the affinity does not change. The increase of folate binding between Days 25 and 50 of pregnancy in allantoic

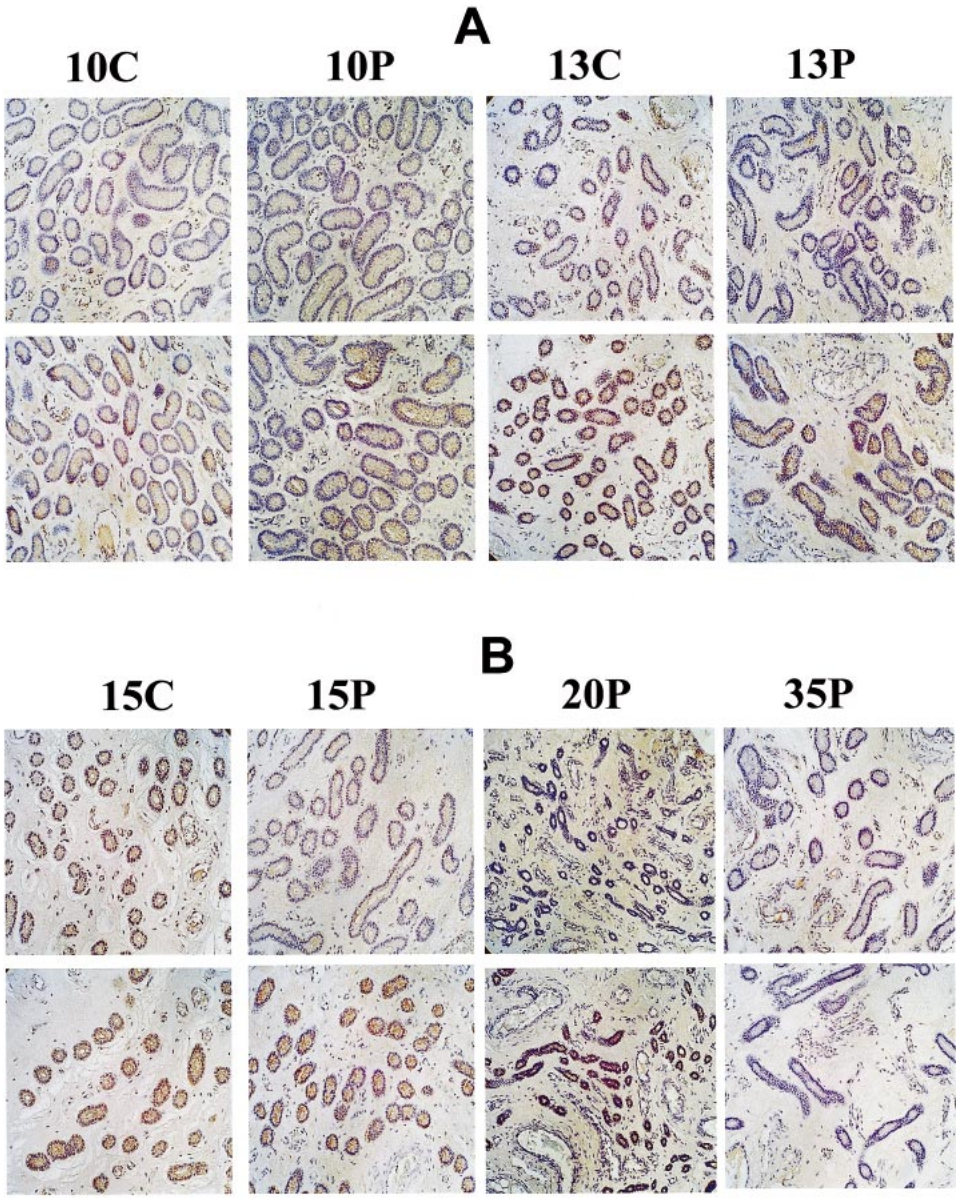

FIG. 4. Localization of sFBP staining in the uterus during the estrous cycle and pregnancy in gilts. In cyclic (C) gilts, light staining for sFBP was present in endometrial glands on Day 10, appeared to be stronger on Day 13, and appeared to decrease by Day 15 (**A**, **B**). In pregnant (P) gilts, light staining for sFBP was present in the endometrial glands on Day 10 and appeared to be stronger on Days 13, 15, and 20. The sFBP staining was absent after Day 20 of pregnancy (**A**, **B**). Tissue sections (**A**, **B**) were incubated with either preimmune rabbit IgG (first and third rows) or anti-sFBP IgG (second and fourth rows). No specific sFBP staining was present in sections from Day 50 to Day 105 (not shown).

fluid coincides with the time when fetal erythropoiesis is being initiated in the liver [6, 21, 22]. It also coincides with the critical period for fetal loss in the crowded uterus, which occurs around Day 40 of pregnancy [22, 23]. The increase of sFBP binding in allantoic fluid between Days 20 and 50 of pregnancy may reflect changes in folate stored in allantoic fluid. The decrease in folate binding in allantoic fluid in later pregnancy suggests that folate may be lacking as conceptus growth accelerates during late pregnancy. A similar pattern occurs for allantoic fluid uteroferrin content [24], and gilts are known to be iron deficient at birth. Whether gilts are also folate deficient at birth remains to be determined.

In placental microsomal membranes, maximum folate binding occurs by Day 50 of pregnancy, and this high level of folate binding is maintained throughout the remainder of pregnancy. By contrast, the affinity is the lowest between Days 50 to 70 and increases thereafter. Increased placental folate binding up to Day 50 of pregnancy may be needed to meet the requirement of the developing conceptus as fetal erythropoiesis is being initiated in the liver around Day 30 and reaches a maximum by Day 50 [6, 21, 22, 25]. Interestingly, folate-binding sites in placental microsomal membranes do not increase after Day 70, but the affinity does increase. Thus, folate transport by the placenta after Day 70 may be increased primarily by the increased affinity of placental binding sites.

How the increase in affinity of placental folate-binding sites occurs is unclear. There are previous results in other species suggesting that membrane-bound folate receptors may be associated with other membrane proteins, including G protein and lyn [26]. Binding of these or other proteins could cause the affinity of the placental folate receptor to increase during late pregnancy. Alternatively, changes in the plasma membrane lipid environment could also affect the affinity of the folate receptor. Finally, two pig membrane-bound folate receptors are known [16, 27]. One was cloned from pig endometrium [16] and the other from pig liver [27]. It is possible that the binding sites reported here are the result of expression of more than one folate receptor gene and that the relationship between the expression of the different folate receptor genes changes during late pregnancy. Determining the causes of the increased affinity of

the placental folate-binding sites during late pregnancy will require further experimentation.

The pattern of sFBP staining localized in the endometrial glands on Days 13 and 15 of both cyclic and pregnant gilts (Fig. 4), which appeared stronger than Day 10, is supported by the data from analysis of folate binding in uterine flushings (Table 1) and from measurements in uterine flushings by both immunoblotting [14] and radioimmunoassay [15]. In pregnant gilts, staining for sFBP was localized to endometrial glands until Day 20 and was absent after Day 20. This pattern of sFBP staining is consistent with the concept that sFBP transports folate to the developing conceptuses until the placenta is formed sometime between Days 20 and 35 of pregnancy, after which placental folate binding takes over folate transport. However, the presence of sFBP in allantoic fluid during later pregnancy is not consistent with this concept. Thus, the lack of staining of sFBP in the endometrium after Day 20 may suggest that sources other than endometrium may be the source of the FBP in allantoic fluid. Another possible source of sFBP is maternal serum [28], but the lack of staining of sFBP inside the blood vessels of the endometrium suggests that maternal serum is not the source of allantoic fluid FBP. Using immunohistochemical analysis, adult liver tissue stains abundantly for sFBP (unpublished observations). Thus, folate-binding sites found in allantoic fluid may derive from fetal liver or perhaps other fetal tissues.

While folate binding in the uterine flushings increased on Days 13 and 15 of the estrous cycle and pregnancy in this study and in previous studies [14, 15], endometrial expression of sFBP mRNA did not change between Days 10 and 15 [16]. Increased intrauterine content of sFBP was hypothesized to be due either to the release of stored sFBP from the endometrium or increased translation of sFBP mRNA [16]. Because the immunohistochemistry data does not indicate significant storage of sFBP on Day 10, the increase in intrauterine sFBP is likely to be due to increased translation of sFBP mRNA.

These results suggest a sequential pattern of folate transport, which differs from that previously reported for the transport of other small molecules. Iron transport via the secretion of uteroferrin is the prototype for the transport of small molecules by endometrial protein secretion in the pig. Secretion of this protein by the endometrium occurs in a biphasic pattern, with increases occurring between Days 10 and 13 and between Days 20 and 40 of gestation [24, 25, 29–31]. Likewise, retinol transport appears to be accomplished via the endometrial secretion of retinol-binding protein throughout pregnancy [24, 32]. The sequential folate transport implicated in this study is the first instance of small molecule transport that appears to occur first by endometrial secretion of a binding protein followed by placental expression of a different binding protein. This is consistent with the concept suggested by Friess et al. [33] that the tall columnar epithelial cells at the top of the folds in placental microstructure may participate in the transport of small molecules. Thus, the sequential nature of folate transport may be the first of many substrates that are delivered in this way, and further research is necessary to specifically explore this possibility. If this is a generalized phenomenon, the shift of transport from endometrial to placental control of transport between Days 20 and 35 of gestation may be susceptible to failure and may explain a portion of the losses that occur due to limitations in uterine capacity during this period [22].

In conclusion, this is the first study describing folate binding in allantoic fluid and placental microsomal membranes throughout pregnancy in swine. The sFBP in the intrauterine environment, which appears to be derived from the endometrial glands, may be the major route of folate transport before placentation. After that, endometrial production of sFBP ends and placental mFBP likely becomes the major transporter of folate, which is reflected by increased folate binding between Days 35 and 50 in placental microsomal membranes. The level of folate binding in allantoic fluid increased from Day 50 to Day 70, after which levels of folate binding decreased. Thus, proper sequential transition of folate transporters during midgestation and increased placental affinity for folate during late gestation may be important for fetal development and reproductive success.

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